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Surface sensing and stress-signalling in *Ulva* and fouling diatoms – potential targets for antifouling: A Review

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Abstract

Understanding the underlying signalling pathways that enable fouling algae to sense and respond to surfaces is essential in the design of environmentally friendly coatings. Both the green alga *Ulva* and diverse diatoms are important ecologically and economically for being persistent biofoulers. *Ulva* spores exhibit rapid secretion, allowing them to quickly permanently adhere to a ship, whilst diatoms secrete an abundance of extracellular polymeric substances (EPS), which are highly adaptable to suit environmental conditions. There is evidence, now supported by molecular data, for complex calcium and nitric oxide (NO) signalling pathways in both *Ulva* and diatoms being involved in surface sensing and/or adhesion. Moreover, adaptation to stress has profound effects on biofouling capability in both types of organism. Targets for future antifouling coatings based on surface sensing are discussed, with an emphasis on pursuing NO-releasing coatings as a potentially universal antifouling strategy.

Keywords

Ulva, diatoms, antifouling coatings, genomics, nitric oxide (NO), calcium signalling.

Introduction

Biofouling of ships is a major problem due to the increased roughness of the hull resulting in drag, which generates a significant fuel penalty (of up to 86% at cruising speed) (Schultz 2007; Callow & Callow 2011). With shipping emissions predicted to increase to a quarter of the world's greenhouse gas output by 2050 (Kennedy et al. 2011), effective antifouling coatings are urgently required. Since the ban of application of the effective but toxic TBT coatings, there has been an increase in the use of copper-based antifouling paints (AF) along with a range of co-biocides (Finnie & Williams 2009). In addition to problems with passive leaching of copper, copper-based coatings require frequent cleaning, which has resulted in copper concentrations in harbours exceeding water quality standards, leading to enhanced regulation (Earley et al. 2014). There is therefore a need for non-biocidal, environmentally-friendly coatings such as the fouling-release coatings – commercially developed coatings based on polydimethylsiloxane elastomers (PDMSe) (Finnie & Williams 2009).

Comprehensive reviews of the current antifouling technologies by Finnie & Williams (2009) and Lejars et al. (2012) conclude that foul-release coatings are the most environmentally friendly option, due to their longevity and reduction in drag leading to a marked decrease in fuel usage. Persistent biofilms (containing diatoms and bacteria) do, however, remain on foul-release coatings, which require cleaning (Hearin et al. 2015; Tribou & Swain 2015) as they can result in a 10-16% power penalty (Schultz 2007; Schultz et al. 2015). However, the cost of cleaning is negligible compared to the fuel cost savings made (Schultz et al. 2011).

The major algal biofoulers are from two evolutionarily distinct groups: green seaweeds and diatoms, which show varying adhesion to the foul-release coatings discussed above. *Ulva* syn *Enteromorpha* (Hayden et al. 2003) is a common green marine alga that is of great economic importance due to (i) it being a cosmopolitan fouler of submerged surfaces such as ships and (ii) its capacity to form 'green tides' – mass accumulations which can have a major economic and ecological effect (eg. Niu et al. 2010). *Ulva* is considered the major macrofouling alga as it is commonly found attached to ship hulls protected with antifouling paints (Callow & Callow 2002). Diatoms are unicellular algae that are omnipresent in aquatic environments and are responsible for 20-40% of global carbon fixation (Field et al. 1998; Yool & Tyrrell 2003), yet they are also the most frequent and successful microalgal foulers of submerged structures (Wetherbee et al. 1998). Spores and young plants of *Ulva* have weak adhesion to foul-release PDMSe (Gunari et al. 2011; Sundaram, Cho, Dimitriou, Weinman, et al. 2011; Cho et al. 2012; Zhou et al. 2014). By contrast, diatom slimes adhere strongly to PDMSe (Holland et al. 2004; Stanley & Callow 2007; Sundaram, Cho, Dimitriou, Finlay, et al. 2011; Cho et al. 2012; Sokolova et al. 2012). The biofilm composition of microfouling communities differs between

foul-release coatings and biocidal antifouling copper-release coatings (Molino et al. 2009; Hunsucker et al. 2014; Muthukrishnan et al. 2014). Foul-release coatings have a greater diversity of diatoms, being more difficult to remove (Hunsucker et al. 2014), which could therefore encourage selection for more tenaciously adherent species of diatoms.

To aid the improved design of AF coatings, a better understanding of the mechanisms by which organisms detect and respond to the properties of a surface is required. In this review, cell signalling and stress responses in relation to surfaces are discussed in *Ulva sp.*, benthic diatoms (the most problematic for foul-release coatings (Hunsucker & Swain 2016)) and *Phaeodactylum tricornutum*, a planktonic diatom that also has a benthic cell morphotype, in which cell signalling has been extensively studied. Future directions for research are suggested with emphasis on the use of genomic data and the new genetic toolkits that are becoming available for algae.

Summary of Ulva biology and life cycle

At spring tide, millions of microscopic swimming spores are released per day by the adult plant, which actively settle, often gregariously, on detection of preferable substrata by secreting a preformed adhesive. The spore itself is pyriform in shape and 7-8 µm long with four anterior flagella that form a dome called the apical papilla, which is thought to be the main 'sensing' area of the spore (Callow et al. 1997) (Figure 1a). Many Golgi-derived electron-dense vesicles containing adhesive are also found in this area (Evans & Christie 1970). At the posterior end of the spore are the nucleus and a single chloroplast. The biology of *Ulva sp.* spores, including their life cycle, ultrastructure and investigations into their adhesive is extensively covered in Callow & Callow (2006).

The process of spore settlement begins when the swimming behaviour of the spore changes from random motion to a more localised searching pattern (Heydt et al. 2007). The spore makes contact with the surface via its apical papilla and then spins, 'sensing' the surface for seconds to minutes until it either permanently adheres or swims off (Callow & Callow 2000). Permanent adhesion occurs when the spore stops spinning and releases its adhesive vesicles over a time-frame of ~2 minutes to form an adhesive pad (Callow et al. 2000). The flagellar axonemes are then withdrawn into the cell and the lipoprotein sheaths discarded (Evans & Christie 1970). Settled spores assume a more spherical shape (Figure 1b) developing a cell wall within 20 min (Callow & Callow 2000), germinating within 24 h and then undergoing cell division to form the new adult plant (sporeling). Use of monoclonal antibodies revealed that the adhesive continues to be produced after settlement (Stanley et al. 1999). The adhesive vesicles contain an *N*-linked glycoprotein which undergoes rapid progressive curing by S-S bond cross-linking, becoming harder over time (Stanley et al. 1999; Humphrey et al. 2005). Analysis of Expressed Sequence Tags (ESTs) from sporulating tissue of *Ulva linza* (ESTs therefore assumed to be present in *Ulva* spores), revealed expressed genes encoding proteins involved in cell wall synthesis and cell-cell adhesion, with similarities to hydroxyproline-rich cell wall proteins found in flowering plants and pherophorins found in the green alga *Volvox* (Stanley et al. 2005).

Sensing of surfaces by Ulva spores

As outlined above, settlement is an active process and much research has been conducted on the settlement cues that motile spores utilize. A list of factors affecting spore settlement, which include salinity, light and colour of coating, are given in Table 1. Three-dimensional tracking of spores has been analysed using digital in-line holography (Heydt et al. 2007; Heydt et al. 2012; Vater et al. 2015), reviewed by Rosenhahn & Sendra (2012). Spores decelerate when in close contact (0-30 µm) with an attractive hydrophobic surface such as FOTS (tridecafluoroctyl-triethoxysilane), which has high spore settlement. The spinning motion of spores appears to be an active behaviour probing the surface, as they only spin for seconds on a surface which has low settlement (AWG – acid washed

glass) compared to minutes on FOTS. It was proposed that the spinning is a way of testing how well a spore adheres to a surface (a small amount of 'temporary adhesive' was seen to be released at this stage using video microscopy (Callow et al. 1997)). Active sensing of the surface requires signalling mechanisms in the cell to relay information. This could involve mechanosensitive channels leading to the activation of a Ca^{2+}-signalling pathway and resulting in release of the adhesive vesicles, which secure permanent attachment (see 'Targeted secretion and calcium signalling in *Ulva* spores').

Many studies have varied surface topography to investigate microtopographic cues for settlement (reviewed by Scardino (2009) and Scardino & de Nys (2011)). Settlement of spores increases in valleys and pillars which are 5 µm deep and 5 µm wide i.e. the same diameter as the spore body when settled – it is thought that this enhances adhesive contact and protects spores from both hydrodynamic forces and desiccation (Callow & Callow 2002; Hoipkemeier-Wilson et al. 2004). Further studies have found that settlement on topographies decreases with increasing aspect ratio (feature height/feature width) (Schumacher, Aldred, et al. 2007). Spore settlement is also reduced on complex topographies inspired by those found on fast moving sharks (Sharklet AF^{TM}) compared to smooth PDMSe (Carman et al. 2006; Schumacher, Carman, et al. 2007), with a reduction in gregarious settlement on the Sharklet surface suggesting that the spores' ability to sense the presence of settled spores is affected (Cooper et al. 2011). Attachment models have been developed to ascertain the effect of the Sharklet features on spore settlement (Long et al. 2010) and on surfaces other than PDMSe (Magin et al. 2011).

QS signalling and its effects on settlement

Spores settle preferentially on top of bacterial biofilms (Joint et al. 2000) and the interactions between bacteria and spores has been investigated, in particular the detection of bacterial quorum-sensing (QS) by spores to select sites for attachment. Gram-negative bacteria, which are predominant in the marine environment, produce *N*-acylhomoserine lactones (AHLs) (Zhang et al. 2006; Williams et al. 2007). When the level of AHLs produced by bacteria reaches a threshold concentration, they bind to a cytoplasmic receptor, which then activates expression of QS genes needed for biofilm formation. *Ulva* can detect and respond to QS signalling pathways involving AHLs (Joint et al. 2002; Tait et al. 2005; Wheeler et al. 2006; Tait et al. 2009). The bacterium *Vibrio anguillarum* stimulated settlement of *Ulva* spores, whereas its AHL-deficient mutant inhibited spore settlement (Joint et al. 2002; Tait et al. 2005). Further studies supported the role of AHLs in spore settlement showing that synthetic AHLs induce spore settlement (Joint et al. 2007) and that AHLs act as a strong chemoattractant, leading to spore deceleration and enhanced settlement in the vicinity of the AHL (Wheeler et al. 2006). Elevations in $[Ca^{2+}]_{cyt}$ occurred in spores in response to AHLs (Joint et al. 2007) and it was postulated that the elevated $[Ca^{2+}]_{cyt}$ acts as a second messenger in response to an unknown receptor resulting in changes in flagellar movement.

Targeted secretion and calcium signalling in Ulva spores

Early studies using electron microscopy revealed the absence of vesicles in settled spores, suggesting that exocytosis had occurred (Evans & Christie 1970). Subsequent video microscopy revealed intense cytoplasmic activity as the spore undergoes permanent adhesion, attributed to the movement of adhesive vesicles for release at the plasma membrane (Callow et al. 1997). Inhibition of secretion and membrane traffic resulted in a 50% reduction in spore settlement (Callow et al. 2001) providing further evidence that exocytosis of adhesive vesicles is required for spore adhesion.

To investigate the secretion process further, Thompson et al. (2007) used the fluorescent styryl dye FM 1-43 to investigate endocytosis – the process of vesicle recovery from the plasma membrane that occurs after exocytosis to prevent cell expansion and regulate membrane recycling (Marcote et al. 2000). If spores undergo mass secretion upon settlement then membrane recycling is needed to prevent expansion of the plasma membrane (Samuels & Bisalputra 1990). In plants, endocytic

retrieval of plasma membrane occurs through clathrin-coated pits and is targeted to a series of endosomal structures that sort the material for recycling (Geldner 2004). Molecular studies of sporulating tissue of *U. linza* have revealed ESTs coding for a clathrin vesicle coat protein (Stanley et al. 2005).

The fluorescent dye FM 1-43 has a high affinity for lipid membranes but cannot penetrate the cytosol, hence intracellular labelling is a marker for plasma membrane entering the cell via endocytosis (Emans et al. 2002). When spores were incubated with FM 1-43, rapid endocytosis occurred at settlement with a discrete spot appearing in the cell within a minute after committing to settlement (Figure 2) (Thompson et al. 2007). The localised nature of FM 1-43 internalisation indicated targeted membrane retrieval to an endosomal compartment. Metabolic pathway analysis of *U. linza* transcriptome data provided further support for membrane recycling with nearly 20% of reads assigned to cellular processes classified as related to endocytosis (X. Zhang et al. 2012). Compared to other plant and algal species studied such as tobacco, broad bean, *Arabidopsis* and the diatom *Coscinodiscus wailesii* (Kubitscheck et al. 2000; Ueda et al. 2001; Emans et al. 2002; Kuhn & Brownlee 2005), *Ulva* spores show rapid secretion and membrane recycling which may explain why it is such an effective fouler as it can adhere to a ship rapidly.

An alternative way to measure secretion is to use carbon-fibre microelectrodes that electrochemically detect release of individual vesicles if they contain redox-active products (Chow et al. 1992). The technique was used with *Ulva* to test whether spores secreted products that were oxidisable upon settlement (Thompson 2007). Although spontaneous release of oxidisable products at the point of spore settlement was not detected, a population of vesicles containing redox-active molecules were found when settled spores were mechanostimulated (Thompson 2007), indicating the presence of mechanosensitive channels in the plasma membrane. Callow & Callow (2011) discussed the possibility of mechanotransducer receptor proteins in the cell membrane that allow the cell to detect the surface topography and induce signalling pathways that modify spore settlement – an essential signalling pathway being the release of the adhesive vesicles.

The regulation of membrane cycling, involving the endomembrane system, cytoskeleton and plasma membrane, is Ca^{2+}-dependent in plants and animals (Steer 1988; Battey & Blackbourn 1993; Barclay et al. 2005) and ESTs coding for Ca^{2+}-signalling related genes such as calcium-dependent protein kinases have been identified in *U. linza* sporulating tissue (Stanley et al. 2005). Therefore, the potential role of Ca^{2+} upon settlement of *Ulva* spores (Figure 3) was studied in Thompson et al. (2007) using fluorescent Ca^{2+}-indicators. Settlement was induced by the addition of low melting point agarose and settling cells showed either transient or prolonged increases in $[Ca^{2+}]_{cyt}$. The spore undergoes several processes upon settlement that may require elevations in $[Ca^{2+}]_{cyt}$ including exocytosis, deflagellation and changes in cell shape. Both secretion and deflagellation are Ca^{2+}-regulated in *Fucus*, *Chlamydomonas* and *Phaeocystis globosa* (Roberts et al. 1994; Chin et al. 2004; Bothwell et al. 2006) and in *Ulva* spores, $[Ca^{2+}]_{cyt}$ elevations occurred at the same point as deflagellation as well as prior to settlement when $[Ca^{2+}]_{cyt}$ was two-fold higher than in settled spores (Thompson et al. 2007). The source of the elevation in $[Ca^{2+}]_{cyt}$ may be extracellular as when Ca^{2+}-influx was blocked using the Ca^{2+} channel inhibitors verapamil and gadolinium, there was a reduction in settlement (Thompson 2007). Internal release of Ca^{2+} from stores such as the ER and vacuole may also be important and can be induced via Ca^{2+}-influx through the plasma membrane (Sanders et al. 1999). Elevation of $[Ca^{2+}]_{cyt}$ appears to be an important trigger for spore settlement, but further studies are needed using inhibitors such as the aminosteroid U73122 which blocks phospholipase C-mediated intracellular Ca^{2+} release (Lee & Shen 1998; Coelho et al. 2002). Fast-Ca^{2+} imaging could also be used to determine whether a single $[Ca^{2+}]_{cyt}$ elevation triggers all the processes or whether each process is regulated by spatio-temporal patterns of $[Ca^{2+}]_{cyt}$. Caged Ca^{2+}-reagents such as Ca^{2+}-

nitrophenyl-EGTA (Ca^{2+}-NP-EGTA), which deliver Ca^{2+} upon photolysis, could be used to transiently elevate $[Ca^{2+}]_{cyt}$ to see if this triggers spores to secrete their adhesive and hence settle.

Role of nitric oxide in spore settlement and adhesion

As previous studies had shown a role for elevated nitric oxide (NO) in reducing diatom, bacterial and animal cell adhesion (Bohl & West 2000; Charville et al. 2008; Thompson et al. 2008; Werwinski et al. 2011), the signalling molecule NO and its effects upon adhesion were studied in *Ulva* spores (Thompson et al. 2010). Artificially elevating NO by the addition of the NO donor SNAP (*S*-nitroso-*N*-acetylpenicillamine) resulted in a 30% reduction in spore settlement and complete removal of spores compared to 45% removal of control cells (without SNAP). Sporeling attachment was also reduced under elevated NO (Thompson et al. 2010). As the reduction in settlement was not due to reduced motility, it was hypothesised that NO may inhibit the signalling process leading to the secretion of the adhesive vesicles. As previously discussed, elevations of $[Ca^{2+}]_{cyt}$ occur at spore settlement and in ventricular cells, NO inhibited the Ca^{2+} current by increasing the second messenger cGMP (cyclic Guanosine MonoPhosphate) preventing Ca^{2+} entry into the cell (Gallo et al. 2001). A similar mechanism could exist in *Ulva* spores with elevated NO reducing the Ca^{2+}-influx so that release of the adhesive vesicles is affected. As sporeling adhesion was reduced when grown in the presence of NO (Thompson et al. 2010), NO may also affect curing of the adhesive such as cross-linking reactions (Humphrey et al. 2005) (Figure 4a).

NO production on surfaces of differing physio-chemical properties was also tested. Spores produced more NO on a surface to which they were weakly attached (Intersleek 900, International Paint), with lower production of NO on polyurethane - a surface which spores attach strongly to (Thompson et al. 2010). It was proposed that the *Ulva* spore uses NO as an intracellular signalling molecule to detect how conducive a surface is for settlement and adhesion by using mechanotransducing receptors to detect how stable they are on a surface. On a hydrophilic surface the adhesive spreads further (Callow et al. 2005) therefore the cell is more stable (Figure 4b). On a hydrophobic surface the adhesive does not spread as far so the cell will be less stable which could initiate a stress response involving NO. The production of NO may be via the enzyme nitric oxide synthase (NOS) (Foresi et al. 2010), or through NADPH-dependent nitrate and nitrite reductases (Yamasaki et al. 1999). A functional NOS has been identified in the green algal *Ostreococcus* genus with 45% similarity to human NOS (Foresi et al. 2010).

Interface between stress responses and biofouling in Ulva

Marine algae naturally produce reactive oxygen species (ROS) as byproducts of photosynthesis and mitochondrial respiration (Marshall et al. 2005). Environmental stresses can cause low levels of ROS to accumulate (McKersie & Lesham 1994). Adaptation methods include enzymatic and non-enzymatic mechanisms to remove the excess ROS. If levels exceed the capacity of the defence systems, then photosystems can be damaged (Dummermuth et al. 2003). There has been extensive research on the stress-adaptation mechanisms used by *Ulva* that have led to it being a successful fouling organism, as outlined below.

Salinity stress

Algae attached to boats will travel through varying degrees of salinity, with lower salinity expected close to the coast where riverine freshwater inputs are high. *Ulva* is often found in brackish estuary waters where it flourishes on the nutrient-rich inputs of rivers and streams. It must therefore have stress-tolerance mechanisms for dealing with changes in salinity. An accumulation of proline and a decrease in $[Ca^{2+}]_{cyt}$ occurred in *U. fasciata* when exposed to extreme salinities (Lee & Liu 1999). Proline protects macromolecules such as proteins and membranes from the effects of stress and acts as a nitrogen storage compound. Oxidative damage was not induced by hypersalinity (90‰) as enzymatic and non-enzymatic defence mechanisms scavenged the excess ROS (Sung et al. 2009).

Activities of the ROS scavenging enzymes superoxide dismutase (SOD), APX (ascorbate peroxidase), catalase (CAT) and glutathione reductase (GR) were all increased during hypersalinity stress in both *U. fasciata* (Sung et al. 2009) and *U. prolifera* (Luo & Liu 2011) and genes encoding SOD, APX and CAT have been identified in *U. linza* through transcriptome analysis (X. Zhang et al. 2012). In *U. prolifera*, extremes of low and high salinity prevent release of spores and decrease rhizoid formation (Dan et al. 2002).

Desiccation stress

Populations of algae situated higher up a ship hull will be prone to desiccation, so would be more similar to intertidal populations than those at the bottom of a hull (comparable to permanently submerged subtidal populations). Ross & Alstyne (2007) studied subtidal vs intertidal populations of *U. lactuca* and found that production of ROS (H_2O_2) occurred between cells following osmotic stress and desiccation by using confocal microscopy to visualize the fluorescent dye DCFH-DA (dichlorodihydrofluorescein diacetate). Intertidal populations were adapted to desiccation stress by producing lower amounts of ROS, being more efficient at removing ROS and by having a higher threshold for oxidative stress. Adaptation mechanisms again include higher activities of the antioxidant enzymes APX, CAT and GR but not SOD as levels were the same between subtidal and intertidal populations (Ross & Alstyne 2007).

Heavy metal stress

Research on the effects of copper (Cu) on *Ulva* is of most interest here as Cu is the main biocide in use again since paints became TBT-free (Chambers et al. 2006). *Ulva* is able to accumulate Cu, with a 40-fold increase in Cu content in plants growing in Cu-contaminated environments compared to control sites (Ratkevicius et al. 2003). Intracellular changes in response to elevated Cu are an increase in vacuolation and the number of electron-dense precipitates contained within the vacuoles, an increase in the number and size of lipid droplets and irregularities in the Golgi apparatus and thylakoid membranes (Andrade et al. 2004). Cu was detected mainly in vacuoles, hence it was concluded that *Ulva* cells reduce Cu toxicity by immobilizing it as a precipitate in vacuoles.

Different species of *Ulva* deviate in their tolerance to Cu (Ratkevicius et al. 2003), however, upregulation of antioxidant enzymes seems to be a common coping mechanism for Cu stress. In *U. fasciata*, oxidative stress is induced by the addition of $CuSO_4$ over 4 days resulting in upregulation of antioxidant enzymes but also denaturation of proteins (Wu & Lee 2008). In *U. compressa*, the effect of elevated levels of Cu is attenuated by activation of the antioxidant enzyme APX, synthesis of ascorbate and consumption of glutathione and water-soluble phenolic compounds (Ratkevicius et al. 2003). *U. compressa* cells exposed to Cu have elevated $[Ca^{2+}]_{cyt}$ and ROS, which determines the differential activation of antioxidant and defence enzymes (Gonzalez et al. 2010). Elevated $[Ca^{2+}]_{cyt}$ binds to and activates a plasma membrane-bound NADPH oxidase resulting in the production of H_2O_2. Signal transduction proteins such as calmodulin and calcium-dependent protein kinase, which are activated in response to elevated $[Ca^{2+}]_{cyt,}$ are upregulated in response to Cu stress (Contreras-Porcia et al. 2011). The Cu-induced $[Ca^{2+}]_{cyt}$ release is due to the activation of NAADP-, ryanodine- and IP_3-sensitive channels and is activated by NO and H_2O_2 (Gonzalez et al. 2012).

Future directions for Ulva studies

As outlined above, the settlement behaviour of *Ulva* spores on surfaces of varying topographies and chemistries has been extensively analysed. Studies on cell signalling have become more common as advanced techniques have become available. Recently there has been a focus on developing *Ulva* as a model organism for use in studies investigating both the evolution of multicellular development and the cross-kingdom interactions between algae and bacteria that influence growth and development in *Ulva* (Wichard et al. 2015). *Ulva mutabilis* and *Ulva linza* can now be grown in axenic

laboratory-based culture (Spoerner et al. 2012; Vesty et al. 2015), which has enabled generation of transgenic *Ulva* for the first time (Oertel et al. 2015) (Table 2). Axenic cultures and the transformation protocol (Oertel et al. 2015) are now readily available and an *Ulva mutabilis* genome-sequencing project is currently underway (Wichard et al. 2015). Once the genome is available, it will be possible to use reverse genetic approaches to knock-out or modify key genes such as those involved in cell adhesion (including those identified from ESTs (Stanley et al. 2005)) to analyse the effects on spore adhesion. The genetic tools are therefore becoming available to further understand the little-studied cell biology of *Ulva*, which should provide further insight into future target areas for antifouling strategies.

Summary of diatom biology and adhesion

Raphid diatoms (Figure 5) lack flagella and move and adhere to a surface only through the secretion of extracellular polymeric substances (EPS) from a slit(s) called the raphe (Round et al. 1990; Wetherbee et al. 1998). Raphid diatoms are the most common early algal colonisers of substrata in seawater where they form the primary biofilm together with bacteria and other algae (Wetherbee et al. 1998). Araphid diatoms such as *Toxarium undulatum* also foul surfaces (Chiovitti et al. 2008), but are not as prevalent as raphid diatoms (Hunsucker et al. 2014). Initial adhesion is an active process requiring activation of adhesion mechanisms to allow binding to the substratum (Edgar & Pickett-Heaps 1983). Mucilage strands are secreted through the raphe, with the driving force for movement being provided by myosin motors attached to actin filaments connected to the mucilage strands by transmembrane connectors (Poulsen et al. 1999). For a comprehensive review of the biology of fouling diatoms including their mechanisms of adhesion and structural properties of the adhesive see Molino & Wetherbee (2008).

There may be several different polymers involved in the process of permanent attachment, or it may involve only one polymer that undergoes different degrees of cross-linking (Wigglesworth-Cooksey & Cooksey 1992; Wustman et al. 1997; Wustman et al. 1998; Chiovitti et al. 2006). Recently a new method for the isolation and extraction of diatom adhesive has been discovered, which results in less than 2% contamination by cellular material (Poulsen et al. 2014). The adhesive comprises predominantly of carbohydrates similar to *Ulva* spore adhesive and the exopolysaccharides of bacterial biofilms but with additional highly hydrophilic amino acids. Two different diatoms were tested (*Craspedostauros australis* [Figure 5c] and *Amphora coffeaeformis*) that differed in their carbohydrate to protein composition - ~4:1 for *A. coffeaeformis* and ~2:1 for *C. australis*. Earlier experiments by Molino et al. (2006) had also shown a difference in the adhesive composition between the two species which may explain why they have differing strength of attachment (Holland et al. 2004). Diatoms are able to vary the composition of their adhesive, with carbohydrates produced by the planktonic and biofilm cells of *Amphora rostrate* being significantly different (Khodse & Bhosle 2010). Abdullahi et al. (2006) also found that the diatom *Phaedactylum tricornutum* varied its carbohydrate production in response to environmental conditions, indicating that there must be a degree of signal perception (see 'Intracellular signalling in diatoms' section) – it is not possible to say specifically that EPS production changed as there is a high likelihood of contamination from intracellular stores of chrysolaminaran due to the methods used (Chiovitti et al. 2004).

Sensing of surfaces by diatoms

A list of factors affecting diatom attachment, including salinity, light and colour of coating are given in Table 1. Recent research has shown that motility of benthic diatoms (*Seminavis robusta* and *Navicula sp.* [Figure 5d]) is silicate-directed (Bondoc et al. 2016), with cell speed and motility increasing in cells under limiting conditions of dissolved silicic acid (dSi) and decreasing upon addition of dSi in a chemokinetic response. Evidence of chemotaxis towards localised hotspots of dSi was also seen indicating that benthic diatoms selectively perceive and navigate towards dSi, which is

an essential resource. When diatoms were exposed to structurally-related dissolved germanium dioxide, cells moved away from the source rather than towards indicating that the specific response to dSi is receptor-mediated. A specific chemotaxic response was also seen in *A. coffeaeformis* to D-glucose with negative chemotaxis to D-mannose (a toxic sugar) and L-glucose (Cooksey & Cooksey 1988).

Studies into bacteria and diatom interactions are covered in a review by Amin et al. (2012) and have not been as extensively studied as in *Ulva*. Alphaproteobacteria such as *Sulfitobacter* and *Roseobacter* are the most prevalent bacteria associated with diatoms (Schafer et al. 2002): these bacteria are also commonly found with *Ulva* (Tait et al. 2009; Spoerner et al. 2012). Buhmann et al. (2011) found that the presence of bacteria affects the adhesion of *Achnanthidium minutissimum*, with no biofilm forming when cells are kept axenically, and Mieszkin et al. (2012) found that mixed biofilms of bacteria enhance attachment of *Navicula incerta*. Windler et al. (2015) describe the identification of a sterile bacterial supernatant that induces capsule- and biofilm formation in the freshwater diatom *Achnanthidium minutissimum* suggesting a role for bacterial-diatom signalling such as through quorum sensing. The development of a reliable bioassay for determining bacterial and diatom interactions is described which will enable further investigation into possible interkingdom signalling molecules (Windler et al. 2015).

The effect of surface topographies on diatom attachment are discussed in depth in the review by Scardino (2009). When four raphid diatom species with differing cell widths were exposed to various surface topographies, cells showed reduced attachment to surfaces where the texture was smaller than the cell width and conversely attachment increased when the texture was larger than the cell width (Scardino et al. 2006; Scardino et al. 2008) (Table 1). Decker et al. (2013) developed the Surface Energetic Attachment model, which correctly predicted that *N. incerta* attachment decreased with decreasing contact area on a pillar pattern. Effective antifouling technologies such as the pillar surface topographies and superhydrophobic surfaces maintain air pockets between the features reducing the potential attachment sites for the diatoms *N. incerta* (Decker et al. 2013) and *A. coffeaeformis* (Wu et al. 2013).

Diatom adhesion to foul-release coatings

Nonbiocidal coatings such as those based on PDMSe have been effective in reducing fouling by macroalgae and invertebrates (Candries et al. 2003). These hydrophobic, low modulus coatings do not prevent colonisation by fouling organisms but are designed as ‘‘fouling-release’’ coatings; that is, they ‘‘release’’ adhered organisms by the hydrodynamic forces generated when a ship moves through the water. Paradoxically, and in contrast to macroalgae such as *Ulva*, diatoms adhere more strongly to hydrophobic coatings and, conversely, adhere more weakly to hydrophilic surfaces such as glass (Holland et al. 2004; Krishnan et al. 2006; Stanley & Callow 2007; Thompson et al. 2008; Alles & Rosenhahn 2015). Diatom genera requiring the highest pressure for removal from foul-release coatings are *Achnanthes*, *Amphora*, *Cocconeis*, *Navicula* and *Synedra* which are all benthic diatoms, centric diatoms do settle but are easily removed in dynamic conditions (Hunsucker & Swain 2016). On organosilica-based xerogels which varied in their surface chemistry (hydrocarbon, fluorocarbon, or aminoalkyl), initial attachment of *Navicula perminuta* was similar but removal increased with increasing critical surface tension and wettability (Finlay et al. 2010).

Recent approaches to testing diatom adhesion have investigated removal of a developed biofilm rather than only diatom cells to make observations more comparable to natural conditions (Hodson et al. 2012; Finlay et al. 2013). Hodson et al. (2012) used a novel turbulent flow channel that allowed direct observation and recording of cell populations on the test surface whilst cells were continually submerged to prevent cellular damage by exposure to air. It was previously shown that cells settled on the hydrophobic surface PDMSe experience stress and cell death when accidentally exposed to

air (Thompson 2007). *C. australis* cells settled on PDMSe were exposed to air by allowing the seawater medium to run off the slide, resulting in a rapid increase in cell death and production of NO, with over 90% cells being dead after 1 min exposure (Thompson 2007). It is therefore important to keep cells hydrated during adhesion experiments using hydrophobic coatings in order to record responses of live, and not dead, cells. Using a newly developed water channel, Hodson et al. (2012) found that when cells were exposed to shear stress for longer periods (180 min cf. 5 min), differences in removal between surfaces of varying wettabilities was reduced. Differences in adhesive properties were also found between three isolates of *A. coffeaeformis* indicating a large degree of adaptability in this species, which may be the reason for their high success rate of adapting and colonising antifouling coatings.

Finlay et al. (2013) also developed a novel biofilm channel to culture diatom cells with the additional variable of changing bed shear stress of 0-2.4 Pa. *Navicula* biofilms were grown for three days and there was no exposure to air during settlement as cells were settled in the same position as they were exposed to flow (although taken out underwater for adhesion strength tests). Adhesion strength of the cultured diatom slime layer, which consisted of both cells and EPS secretions, was higher than individual cells on clean surfaces. When cells were cultured under a shear stress of 1.3 Pa on AWG the biofilm did not persist, yet on PDMSe a biofilm was still present at 2.4 Pa containing tightly bound clumps and a greater proportion of cells lying on their valve (raphe side), which is thought to be hydrodynamically favourable.

Alles & Rosenhahn (2015) tested the use of a microfluidic detachment assay commonly used in cell biological tests. The benefits of such a system are that it requires only a small number of cells, along with a small test surface area, with which a high variation in well-defined laminar flow conditions can be tested. The design also allows for cell adhesion to be tested without passing through the air-water interface, with individual cells examined microscopically in situ whilst exposed to flow conditions. By studying removal microscopically it was possible to observe whether cells lying on their valve detached more easily than those on their girdle or vice versa. Again, more *Navicula* cells lay on their valve (therefore the raphe is in contact with the surface) on a hydrophobic surface compared to a hydrophilic one (43% on the hydrophobic DDT compared to 30% on the hydrophilic EG_6OH), however there was no significant effect of cell orientation in the critical shear stress required to remove cells (Alles & Rosenhahn 2015), which may be due to the high degree of variability in adhesion strength seen.

Intracellular signalling in diatoms

It has only recently become recognised that diatoms have sophisticated cell signalling pathways allowing them to detect and respond to physiochemical changes in their environment.

Cytosolic calcium

An early study identified Ca^{2+} as being essential in diatom adhesion but did not identify whether it was extracellular or $[Ca^{2+}]_{cyt}$ that was important, or both (Cooksey 1981). As $[Ca^{2+}]_{cyt}$ is involved in so many cellular activities (Berridge et al. 1998) including exocytosis, which is essential for transport of adhesive vesicles containing EPS to the raphe in raphid diatoms (Wetherbee et al. 1998), further studies investigated the role of $[Ca^{2+}]_{cyt}$. Use of a Ca^{2+} transport inhibitor (D-600) that reduced adhesion suggested that there is an intracellular role for Ca^{2+} (Cooksey & Cooksey 1986). Advances in the use of transgenic diatoms, specifically *P. tricornutum*, have confirmed that Ca^{2+} signalling controls many aspects of diatom function (Falciatore et al. 2000), as it does in plants (Valmonte et al. 2014). Falciatore et al. (2000) developed transgenic *P. tricornutum* containing the Ca^{2+}-sensitive photoprotein aequorin and discovered various Ca^{2+}-sensitive responses to shear stress, osmotic stress and iron. McLachlan et al. (2012) used fluorescent AM-ester Ca^{2+}-indicators to investigate $[Ca^{2+}]_{cyt}$ signalling in relation to phototaxis in *N. perminuta*, which has been well studied for its

adhesion to fouling surfaces (McLachlan et al. 2012). The photophobic response seen after exposure to high irradiance blue light (450-490 nm) was triggered by a localised, transient increase in $[Ca^{2+}]_{cyt}$ at the tip of the cell, implying a role for Ca^{2+} signalling in the switching mechanism required for cell reversal. However, further investigations using dextran dyes introduced by biolistic bombardment (as used in *P. tricornutum* in Falciatore et al. (1999)) would be beneficial to ensure that the increases in dye fluorescence are due to changes in $[Ca^{2+}]_{cyt}$ and no other factors (see Thompson et al. (2007) for problems encountered when using AM-ester Ca^{2+}-indicators in algae). Using intracellular Ca^{2+} release inhibitors and Ca^{2+} channel inhibitors it was shown that the source of $[Ca^{2+}]_{cyt}$ was from intracellular stores and not due to influx of extracellular Ca^{2+} (McLachlan et al. 2012). Also of interest was the localisation of $[Ca^{2+}]_{cyt}$ in the vicinity of the raphe in motile cells where the actin/myosin motility system is located, supporting a role for Ca^{2+} in generating motive force in diatoms. Enhanced NO production was also seen in the vicinity of the raphe of *S. robusta* (Thompson et al. 2008) where it could affect motility and adhesion (see later section 'Nitric oxide').

Ca^{2+}-signalling pathways are important in cells of *P. tricornutum* in response to growth in Fe-limiting conditions, with upregulated mRNA levels of Ca^{2+} protein kinase, Ca^{2+} channels and the oxidative stress responsive protein annexin (Allen et al. 2008). Further evidence for Ca^{2+}-signalling pathway components has been found from the diatom EST database with ESTs relating to Ca^{2+} transporting ATPase, annexin and domains containing Ca^{2+}-binding EF hands having been identified (Maheswari et al. 2010) (Table 2). Ethylenediaminetetraacetic acid (EDTA) is a chelating agent which sequesters Ca^{2+} and other metal ions such as Fe^{3+}. Chiovitti et al. (2008) found that adhesion is prevented in *T. undulatum* by the addition of EDTA, which destabilises the adhesive proteins. The adhesive nanostructure could be restored by the addition of cations including Ca^{2+} therefore Ca^{2+} may act to cross-link and stabilise the glycoproteins in the adhesive. In *P. tricornutum*, a putative cell adhesion protein has been identified containing Ca^{2+}-binding domains, which was also proposed to have a role in cross-linking (Willis et al. 2014). However, none of the cell adhesion proteins identified in Willis et al. (2014) were shown to be present in the adhesive and therefore their precise role in diatom adhesion remains unknown. Another component of eukaryotic Ca^{2+} signalling pathways appears to be conserved in diatoms with the discovery of a G-protein coupled receptor that responds to environmental stress and effector proteins including phospholipase C and protein kinase C all being present in *P. tricornutum*, *T. pseudonana* and the raphid diatom *Fragilariopsis cylindrus* (Port et al. 2013).

Stress responses in diatoms and their link to biofouling

Salinity stress

At elevated salinities *P. tricornutum* increases production of EPS and modifies its composition to include a higher proportion of uronic acids and sulphates, which may allow the EPS to retain more water (Abdullahi et al. 2006). Steele et al. (2014) have shown that diatom EPS plays a protective role in response to salinity stress in *Cylindrotheca closterium*, which could explain the increased production of EPS under salinity stress seen in *P. tricornutum*. De Martino et al. (2011) found that changes in salinity and temperature caused *P. tricornutum* to change morphotype, with stressful conditions inducing a change from fusiform or triradiate to oval or round cells. The oval morphotype is benthic, possessing a raphe enabling it to move and form biofilms, whereas the other morphotypes are planktonic. By converting to the biofilm-forming morphotype cells will be more protected from salinity stress, as cells in a biofilm are surrounded by EPS. Examination of expressed sequence tags (ESTs) showed that hyposaline, low temperature conditions induced genes involved in stress signalling pathways (De Martino et al. 2011).

Response to heavy metals

As well as diatoms being persistent foulers of foul-release coatings, they are also a problem on Cu-containing AF paints due to their tolerance to heavy metals. Diatoms that are tolerant to Cu, such as

Amphora and *Navicula*, possess Cu-sequestering intracellular bodies that maintain low concentrations of free Cu^{+} in the cells (Daniel & Chamberlain 1981). An additional method of tolerating high Cu levels in *A. coffaeaformis* is through complexing of EPS polysaccharides with Cu (Robinson et al. 1992).

Stress responses in diatoms in response to heavy metals have not been as extensively studied as in *Ulva* and have focused on stress induced by limited iron bioavailability. The effects of Cu on *P. tricornutum* however have been investigated (Morelli & Scarano 2004), with glutathione and its derived peptides (phytochelatins [PC]) forming the first line of defence by binding free Cu^{+} ions intracellularly. Prolonged exposure leads to the activation of antioxidant enzymes (similar to that seen in *Ulva*) with CAT being the major scavenging enzyme although other enzymes (SOD and GR) also counteracted the oxidative stress induced by Cu. Prolonged Cu exposure induces membrane damage. Genes encoding superoxide dismutase (MnSOD) have been discovered in both *T. pseudonana* (Wolfe-Simon et al. 2006) and *P. tricornutum* (Vardi et al. 2008).

Nitric oxide signalling

Cell biological studies on the diatom *P. tricornutum* using fluorescent imaging discovered that $[Ca^{2+}]_{cyt}$ together with NO were the basis of a sophisticated stress surveillance system (Vardi et al. 2006). Elevations of $[Ca^{2+}]_{cyt}$ from an intracellular source occurred after exposure to the diatom-derived reactive aldehyde (2E,4E/Z)-decadienal (DD). The resulting stress response involves the production of NO via Ca^{2+}-dependent Nitric Oxide Synthase-like activity, which either results in cell death or acclimation of the response. It was proposed that diatom cells can detect that neighbouring cells are stressed by sensing DD released by wounded cells. Calcium and NO-based signalling systems are then sensitised to induce resistance to further aldehyde exposure. In bloom conditions, the aldehyde concentrations reach a threshold, which then triggers population-level cell death and bloom termination.

As NO signalling is important in diatom sensing systems, studies were carried out with the benthic diatom *S. robusta* to investigate whether there was differential production of NO on surfaces of varying physio-chemical properties (Thompson et al. 2008). NO was also artificially elevated using an NO donor to investigate the effects of NO on diatom adhesion. Measurements of NO made using the fluorescent NO indicator DAF-FM showed that different NO levels are seen in response to hydrophobic or hydrophilic surfaces (Figure 4c). On hydrophilic AWG where the adhesion strength of diatoms was low, NO production was 4-fold higher than on PDMSe, to which the cells adhered strongly. Interestingly, the converse was seen for spores of *Ulva*, with lower NO production on AWG, a surface to which they bind strongly (Thompson et al. 2010). Therefore it was hypothesised that both diatoms and spores of *Ulva* can detect a surface that is non-conducive to adhesion through the production of NO which can then modify the adhesive properties (by blocking adhesive production or leading to the production of a less sticky adhesive) making it easier for a diatom to move off the unfavourable surface, or in the case of *Ulva* spores prevent them from committing to permanent adhesion to a surface (Figure 4).

Vardi et al. (2008) provided support for the hypothesis that NO plays a key role in surface perception in diatoms. A functional genomics approach was used to characterise a novel GTP-binding protein designated *Pt*NOA (an orthologue of *At*NOA in *Arabidopsis*) that is involved in NO production in *P. tricornutum.* When transgenic diatoms overexpressed the gene, NO production was elevated and oval morphotype cells showed reduced biofilm formation and reduced adhesion to both AWG and PDMSe. As the aldehyde DD leads to the production of NO in *P. tricornutum* and elevated NO reduces diatom adhesion, Leflaive & Ten-Hage (2011) investigated the effect of DD and NO on adhesion in the freshwater raphid diatom *Fistulifera saprophila*. Incubation with DD and an NO donor both reduced cell adhesion. The authors proposed that DD has an effect on the cellular

mechanisms involved with initial adhesion such as the cytoskeleton rather than secondary processes such as cross-linking (Vreeland et al. 1998), as the addition of DD to adhered cells did not reduce adhesion. As DD is produced by damaged cells, it may act as an environmental signal preventing cell attachment to unsuitable habitats, a point that may be of interest to those developing environmentally friendly coatings (see 'Antifouling targets in relation to surface sensing and stress-signalling').

Future directions for diatom studies

In the post-genomic era, planktonic rather than benthic diatoms were initially the focus of genomic studies as the centric species *T. pseudonana* is used as a model for diatom physiology studies and has a relatively small genome. Molecular tools are now available for reverse genetics enabling gene knockouts in diatoms (De Riso et al. 2009; Lavaud et al. 2012; Nymark et al. 2016). A diatom EST database has been constructed for *P. tricornutum* and *T. pseudonana* grown under different growth conditions and subject to various abiotic stresses (Maheswari et al. 2009; Maheswari et al. 2010); and whole genome arrays of both diatoms have been sequenced (Armbrust et al. 2004; Bowler et al. 2008) (Table 2), all of which should enable diatom cell biology to proceed with enhanced resolution. An example of using genome information to investigate diatom cell biology is the use of transgenic *P. tricornutum* to study membrane trafficking. Both *P. tricornutum* and *T. pseudonana* contain homologs of two vesicle transport proteins – the small GTPase Sec 4 and syntaxin (t-SNARE). Transgenic fusiform *P. tricornutum* with Sec4- and t-SNARE-fluorescent protein fusions were created to image vesicle movement (Siaut et al. 2007; A. Tanaka et al. 2015). If these same marker lines were used in cultures selected for the oval benthic diatom form then it would be possible to investigate membrane trafficking of adhesive vesicles to the raphe. Indeed, a recent study by Willis et al. (2014) used the oval, adherent form of *P. tricornutum* to test adhesion of transformed cells. A bioinformatics search of the genome was used to suggest candidate genes involved in cell adhesion. Cells were then transformed with overexpression of target genes-fluorescent protein fusions to enable localisation of the gene. All of the transgenic lines had improved cell adhesion compared to wild-type, implying that the genes identified may have a role in cell adhesion, however this requires further investigation such as the use of immuno-techniques and the generation of knock-out transformants.

Genomic research on raphid diatoms is becoming more common with the genomes of *Fistulifera solaris*, *Fragilariopsis cylindrus* and *Pseudo-nitzchia multiseries* having been sequenced (Strauss 2012; T. Tanaka et al. 2015; http://genome.jgi.doe.gov/Psemu1) (Table 2). *S. robusta* is currently being fully sequenced (chloroplast sequencing is described in Brembu et al. (2014)) and techniques for genetic transformation of *A. coffeaeformis* have been developed (Buhmann et al. 2014), which will enable the cell biology of fouling diatoms to be fully unravelled in the future.

Now that more algal genomes are becoming available, there is increasing interest in linking studies at the genome level to the organism response (Mock et al. 2016). To investigate this, an upcoming area of interest is in experimental evolutionary studies where semi-continuous cultures of algae are used to study evolution in real time, a technique usually used to study bacterial evolution. The rapid generation time of the model green alga *Chlamydomonas reinhardtii* allowed experimental evolution experiments to select for changes in the production of extracellular matrix, leading to the appearance of multicellularity in only 219 days (Ratcliff et al. 2013). The technique was also used to study real-time evolution in the diatom *Skeletonema marinoi* (Scheinin et al. 2015) where adaptation to high CO_2 resulted in increased growth rates, revealing that evolution can occur rapidly in marine diatoms. It may therefore be possible in the future to examine the genomic response of raphid diatoms to various antifouling coatings by using similar techniques to search for any adaptations to coatings.

Antifouling targets in relation to surface sensing and stress-signalling

For an antifouling molecule to have an effect on a wide range of fouling organisms, it should be something that inhibits a universal process such as surface sensing and/or the signalling pathways that transduce the surface signal (Cooksey et al. 2009). There are four key areas that this review has focused on; Ca^{2+}, stress, quorum sensing and NO.

Calcium

Adhesion in diatoms is Ca^{2+}-dependent (Cooksey & Cooksey 1980; Cooksey 1981) with adhesion strength reduced in *Amphora* when Ca^{2+} entry into the cell was blocked (Cooksey et al. 2009) and motility was also reduced in *N. perminuta*. As *Ulva* spores require Ca^{2+} to move and use of Ca^{2+}-channel inhibitors reduced motility and subsequent settlement, it was not possible to investigate the effect of Ca^{2+} on settlement alone. However, the transient increases in $[Ca^{2+}]_{cyt}$ seen at settlement imply a role for Ca^{2+} in the adhesion of *Ulva* spores (Thompson et al. 2007). Interfering with Ca^{2+} signalling such as through the addition of Ca^{2+}-channel inhibitors to foul-release coatings is therefore likely to reduce adhesion of both algal spores and raphid diatoms.

Stress

Another potential antifouling strategy is to incorporate molecules that trigger stress responses into a coating. One such chemical is DD, which is available commercially in synthetic form and elicits NO generation in diatoms. DD reduces adhesion and thus allows diatoms to leave an unfavourable surface, in addition to inducing cell death at higher concentrations. DD caused loss of motility in the benthic diatoms *Amphora*, *Navicula* (Cooksey et al. 2009) and *F. saprophila* (Leflaive & Ten-Hage 2011), and a significant reduction in *F. saprophila*, *Nitzchia palea* and *Mayamea atomus* biofilm formation occurred when DD was present in the agar (Leflaive & Ten-Hage 2011). Unfortunately, the effect of DD does not appear to be universal in fouling organisms. Whilst addition of DD results in reduced cell growth leading to cell death in the diatom *Thalassiosira weissflogii* (Casotti et al. 2005), and reduces cell division of marine invertebrate embryos (Ianora et al. 2006), it appears that marine bacteria are able to show remarkable resistance to the aldehydes in comparison to that shown by algae (Ribalet et al. 2008). In addition, DD is a toxic chemical which has been shown to affect non-target organisms leading to reproductive failure in the polychaete *Nereis virens* (Caldwell et al. 2011), which is used in aquaculture (Olive 1999). Therefore full environmental testing of the compound with toxicity assays would be required before DD could be incorporated into antifouling coatings to prevent a repeat of the problems with toxic coatings such as occurred with TBT.

Quorum Sensing

QS inhibitors are another area of interest for interfering with cell signalling, this time between bacteria and algae. A commercially available QS inhibitor is kojic acid, which in addition to inhibiting the formation of a marine biofilm by 3-fold was also found to reduce density of *A. coffeaeformis* cells by ~4 fold (Dobretsov et al. 2011). Whether kojic acid is interfering with QS signalling or causing a reduction in fouling by another method is not yet known; however the compound would seem a promising AF strategy. Unfortunately, disruption of AHL-signalling would not necessarily reduce algal spore fouling. Although AHL-deficient mutant biofilms inhibited spore settlement (Joint et al. 2002; Tait et al. 2005), spore germination and growth in the absence of AHLs is enhanced (Twigg et al. 2014), which would result in increased drag on a ship's hull.

Nitric oxide

A potentially more universal application to interfering with cellular sensing mechanisms would be to tether NO donors to antifouling coatings. Artificially elevating NO in diatoms and *Ulva* reduces both settlement and adhesion (Thompson et al. 2008; Thompson et al. 2010; Leflaive & Ten-Hage 2011). NO also weakens adhesion strength of bacteria (Charville et al. 2008; Werwinski et al. 2011) and animal cells such as platelets (Bohl & West 2000). There has been an explosion in interest in the use

of NO as a bacterial biofilm dispersal agent for surfaces in both clinical and industrial environments to control bacterial infections (Firoved et al. 2004; Barraud et al. 2006; Plate & Marletta 2012; Marvasi et al. 2014), with addition of an NO donor resulting in dispersal of fouling marine bacterial biofilms containing *Pseudoalteromonas* species (Werwinski et al. 2011). NO donors are also effective in reducing barnacle cyprid settlement (Y. Zhang et al. 2012) with NO implicated in the regulation of cement release in *Amphibalanus* (Gallus et al. 2013; Zhang et al. 2015), and the potential antifouling compound Cochliomycin A has been reported to reduce larval settlement rates of cypris larvae by activating the NO/cGMP pathway (Wang et al. 2016).

Conclusion

A number of potential areas for interfering with cell surface sensing and signalling mechanisms have been identified in this review. The most promising areas to focus on are the addition of Ca^{2+}-channel inhibitors, the diatom-derived aldehyde DD, QS inhibitors and NO donors. These bioactive chemicals would need to be tethered to foul-release coatings with a self-polishing coating so that the bioactive surface is constantly replenished. Majumdar et al. (2008) used such a system with quaternary ammonium salts tethered to polysiloxane coatings, which also had foul-release characteristics. There are a number of factors that would need to be investigated for such coatings to be successful such as; any negative effects on non-target organisms including humans and marine organisms, a highly controlled release rate, cost effectiveness of the coatings, necessity for cleaning, and durability and stability in seawater. See Banerjee et al.(2011) for a review of the areas required for consideration in designing antifouling coatings including discussion on covalent-linking of NO-donors to a siloxane polymer. The authors believe a NO-releasing coating should be investigated as there is the potential for a universal antifouling strategy that would reduce settlement and adhesion in algae, barnacles and bacteria. As the cell biology of fouling organisms is still relatively unknown and an area that is likely to yield new information in the post-genomic era, there is great potential for new antifouling targets to be discovered.

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Table 1 Factors that affect *Ulva* spore settlement and diatom adhesion.

	Ulva sp		**Diatoms**	
	Effects	**References**	**Effects**	**References**
Surface properties				
Wettability	Increasing spore settlement with increasing contact angle (hydrophobicity). Spore adhesion strength is greatest on a hydrophilic surface.	M.E. Callow et al. 2000 Finlay et al. 2002 Finlay, M.E. Callow, et al. 2002 Ista et al. 2004	No effect of wettability on initial settlement of diatoms. Adhesion strength is greatest on a hydrophobic surface.	Finlay, M.E. Callow, et al. 2002 Holland et al. 2004
Topography	Spores choose to settle in depressions and corners with a feature size similar to that of the spore body (ca. 5µm). Complex topographies reduce spore settlement.	Hoipkemeier-Wilson et al. 2004 Carman et al. 2006 Schumacher, Carman, et al. 2007	Reduced diatom attachment to surfaces where the texture was smaller than the cell width. Diatom attachment increases with increasing numbers of attachment points on a surface.	Scardino et al. 2006 Scardino et al. 2008 Decker et al. 2013
Salinity	Increased settlement at higher salinities up to an optimum (25-30‰ in *U. intestinalis*).	Christie & Shaw 1968	Increasing salinity results in increased EPS production.	Abdullahi et al. 2006
Temperature	Increased settlement at higher temperatures up to an optimum temperature (of 25-30 ˚C in *U. intestinalis*).	Christie & Shaw 1968 Callow et al. 1997	Species-specific effects on adhesion. Increased temperature causes increased motility up to 35-40˚C, beyond which motility stops.	Cohn 2001
Light	Increased initial settlement rate in daylight but it is not essential for settlement.	Christie & Shaw 1968	Increased EPS production in darkness. High intensity light triggers cell reversal therefore cells move away to lower intensity light. Light from below inhibits settlement of *Navicula.*	Smith & Underwood 1998 Cohn et al. 2004 Cao et al. 2011
Colour of coating	Spores prefer to settle on black surfaces rather than white. Sporeling growth is delayed on black surfaces.	Swain et al. 2006	Including a glow-in-the-dark phosphor layer in an antifouling paint reduces initial settlement density of *Navicula*.	Cao et al. 2013
Presence of bacteria	Bacterial biofilms of mixed species enhance spore settlement but biofilms of single species can either attract, inhibit or have no effect on settlement.	Joint et al. 2000 Patel et al. 2003 Mieszkin et al. 2012	Bacterial biofilms of mixed species enhance attachment of *Navicula*. Cells of *Achnanthidium minutissimum* do not adhere when kept axenically.	Mieszkin et al. 2012 Buhmann et al. 2011

Table 2 State of our molecular knowledge of fouling alga including the green alga *Ulva* and various planktonic and raphid diatoms. In addition, the Marine Microbial Eukaryote Transcriptome Sequencing Project has sequenced diatom transcriptomes, which are publicly available at: http://marinemicroeukaryotes.org/

Organism	**Genome**	**Transcripts/EST**	**Transformation**
Ulva linza	X	✓ Stanley et al. (2005) X. Zhang et al. (2012)	X
Ulva mutablis	✓ In progress (Wichard et al. 2015)	X	✓ Oertel et al. (2015)
Planktonic diatoms			
Chaetoceros gracilis	X	X	✓ Ifuku et al. (2015)
Cyclotella cryptica	✓ Traller et al. (2016)	✓ Traller et al. (2016)	✓ Dunahay et al. (1995)
Thalassiosira oceanica	✓ Lommer et al. (2010) Lommer et al. (2012)	X Lommer et al. (2012)	X
Thalassiosira pseudonana	✓ Armbrust et al. (2004)	✓ Maheswari et al. (2009)	✓ Poulsen et al. (2006) Shrestha & Hildebrand (2015)
Raphid diatoms			
Amphora coffeaeformis	X	✓ Buhmann et al. (2014)	✓ Buhmann et al. (2014)
Cylindrotheca fusiformis	X	X	✓ Fischer et al. (1999)
*Fistulifera saprophila (*syn *Navicula saprophila)*	X	X	✓ Dunahay et al. (1995)
Fistulifera solaris	✓ T. Tanaka et al. (2015)	X	✓ Muto et al. (2013)
Fragilariopsis cylindrus	✓ Strauss (2012)	✓ Mock et al. (2005)	X
Phaeodactylum tricornutum	✓ Bowler et al. (2008)	✓ Maheswari et al. (2009)	✓ Apt et al. (1996)
Pseudo-nitzchia arenysensis	X	✓ Di Dato et al. (2015)	✓ Sabatino et al. (2015)
Pseudo-nitzchia delicatissima	X	✓ Di Dato et al. (2015)	X
Pseudo-nitzchia multiseries	✓ http://genome.jgi.doe.gov /Psemu1/Psemu1	✓ Boissonneault et al. (2013)	X
Pseudo-nitzchia multistriata	✓ In progress (Ferrante unpublished)	✓ Di Dato et al. (2015)	✓ Sabatino et al. (2015)
Seminavis robusta	Chloroplast (Brembu et al. 2014)	X	✓ Kirupamurthy (2014)

Figure 1 (a) Diagrammatic view of a motile *Ulva* spore illustrating key features. The smaller image shows the swimming spore and its four flagella. c = chloroplast, e = eyespot, p = pyrenoid, m = mitochondria, n = nucleus, g = Golgi body, v = vesicles containing adhesive, f = flagellum, ap = apical papilla. **(b)** Swimming and settled *Ulva* spores at *t* = 0 (left) and *t* = 5 min (right). Those marked with a black arrowhead are still swimming whilst the rest of the cells have already settled. Note the presence of flagella in motile cells (white arrowheads) and the spherical nature of the settled cells compared to motile cells, which are more elongate.

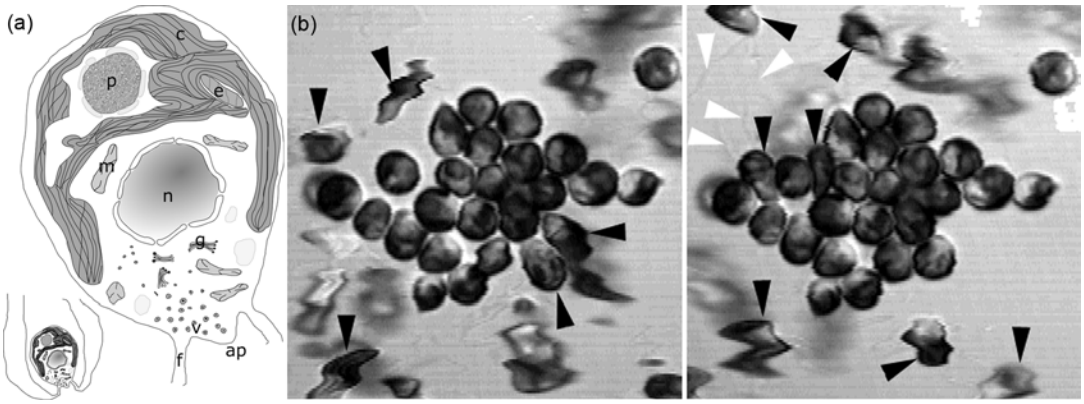


Figure 2 Localisation of FM 1-43 labelling in swimming and settled spores indicating membrane internalisation via endocytosis. **(a)** Confocal images of swimming spores incubated in the presence of FM 1-43 for 3 min. The distorted lines indicate that the cells are moving. Only the plasma membrane is labelled with FM 1-43. Bar = 10 µm. **(b)** Spores that have settled whilst incubated with FM 1-43 for 15 min. All settled spores have an interior cytoplasmic spot of FM 1-43 fluorescence. Bar = 20 µm. **(c)** A settling spore with flagella (arrows) present at *t* = 0. At *t* = 1 min the flagella have been lost and a spot of internalised dye (arrowheads) has formed at the anterior region which is more pronounced at *t* = 5 min. Figure reproduced with permission from Plant, Cell & Environment (John Wiley and Sons).

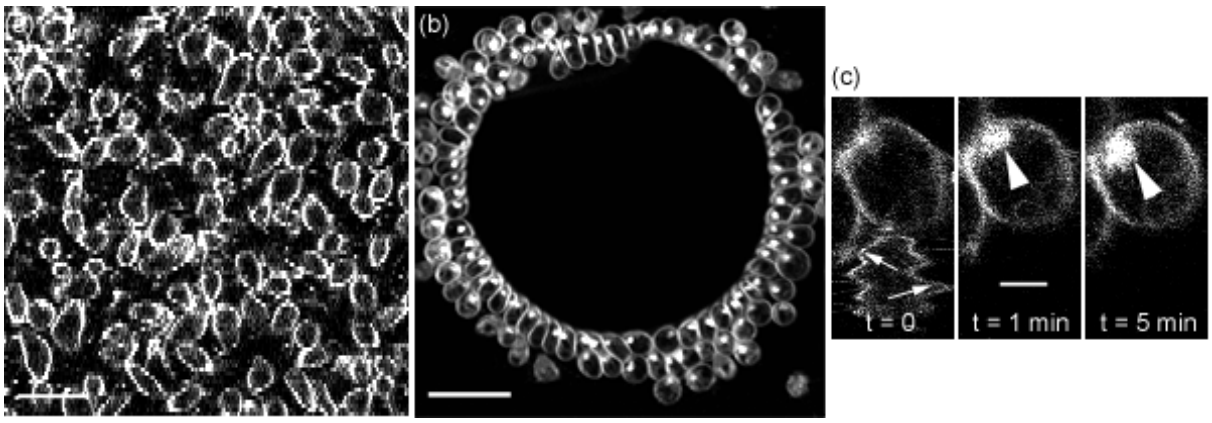


Figure 3 Proposed models for the role of elevated cytosolic Ca^{2+} at spore settlement. **1)** The swimming spore is attracted to a surface by several cues. **2)** The spore temporarily binds and explores the surface rotating on a detachable pad on its apical papilla. The favourable signals cause Ca^{2+} channels to open in the plasma membrane allowing Ca^{2+} to flood in. **3)** Microtubule hypothesis: Ca^{2+} acts directly to activate the transport of the adhesive vesicles possibly along microtubules/microfilaments and allows fusion of vesicles with the plasma membrane resulting in the formation of the adhesive pad **(6)**. **4)** Signal transduction hypothesis: Ca^{2+} acts indirectly by binding with a Ca^{2+} binding protein such as calmodulin. **5)** The Ca^{2+} binding protein activates protein kinases to start a signalling cascade resulting in the release of adhesive vesicles to form an adhesive pad **(6)**. Internal release of Ca^{2+} may also be important, and can be triggered by Ca^{2+} influx through the plasma membrane.

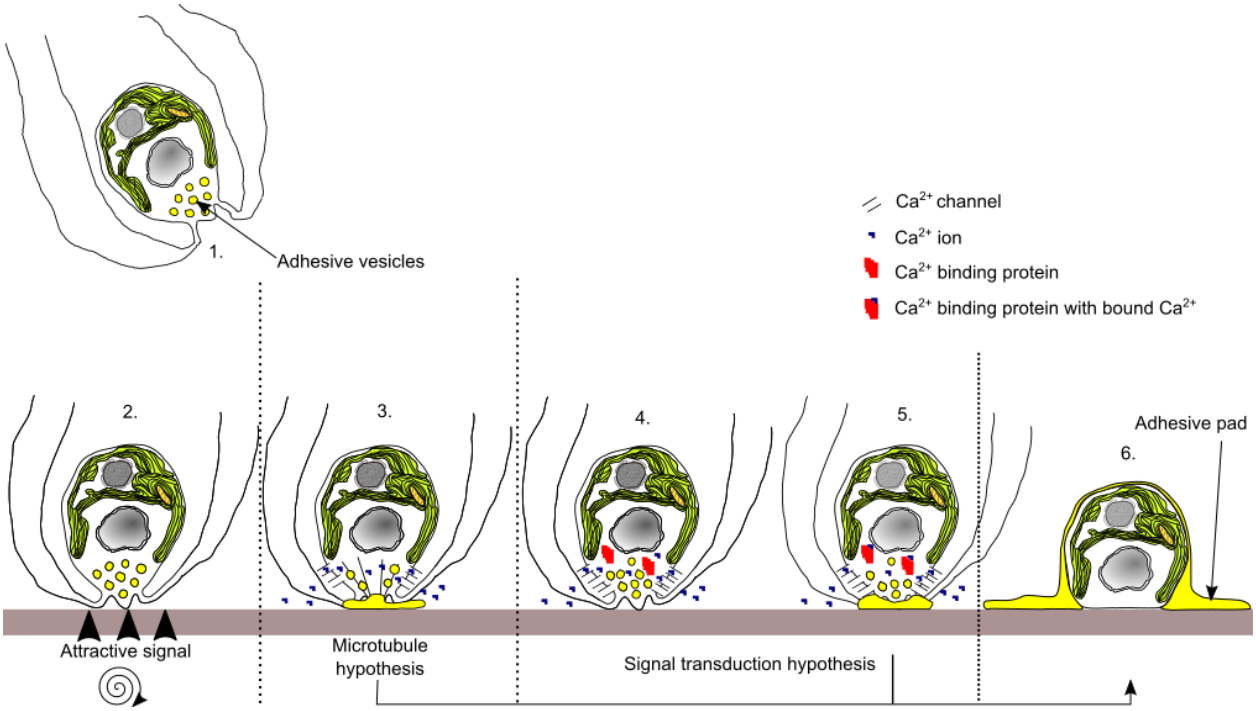


Figure 4. Models for the possible action mechanisms of nitric oxide (NO) in relation to surfaces in *Ulva* spores **(a&b)** and the diatom *Seminavis* **(c)**. **(a)** Spores are less likely to settle in the presence of high NO. Those spores that do settle are removed more easily from the substratum as the adhesive fails to cure. Spores that settle under normal NO levels release adhesive which undergoes full curing involving cross linking reactions. The spore adhesive is then firmly adhered to the substratum and not easily detached when exposed to flow. NO readily diffuses out from cells so will be present in the extracellular medium. **(b)** On a hydrophobic surface (such as Intersleek 700, contact angle 109˚), the spore releases adhesive but it does not spread far. The spore may sense this through mechanotransducing receptors leading to an increase in the production of NO. Spores settled on hydrophobic surfaces are easier to remove. On a hydrophilic surface (such as polyurethane, contact angle 45˚), the spore releases adhesive and it spreads easily. There is no signal sensed that the surface is stressful so extra NO is not produced. Spores settled on a hydrophobic surface are difficult to remove when exposed to flow. **(c)** *Seminavis* detects the adhesiveness of a surface through an unknown signal. On a surface that the cell does not adhere to strongly (hydrophilic), the signal causes the production of NO in the cell. NO then either blocks the secretion of adhesive, making the cell more likely to detach and move off the surface, and⁄or NO causes the production of a less sticky adhesive that is more conducive to movement, resulting in reduced attachment strength. When *Seminavis* is adhered to a surface that it sticks to well (hydrophobic), there is constitutive production of NO that is necessary for growth but NO is not significantly elevated and therefore the cell does not move off the surface. When NO is elevated due to the addition of the NO-donor SNAP, adhesion is reduced through the same pathways as on a hydrophilic surface. SNAP, *S*-nitroso-*N*-acetylpenicillamine.

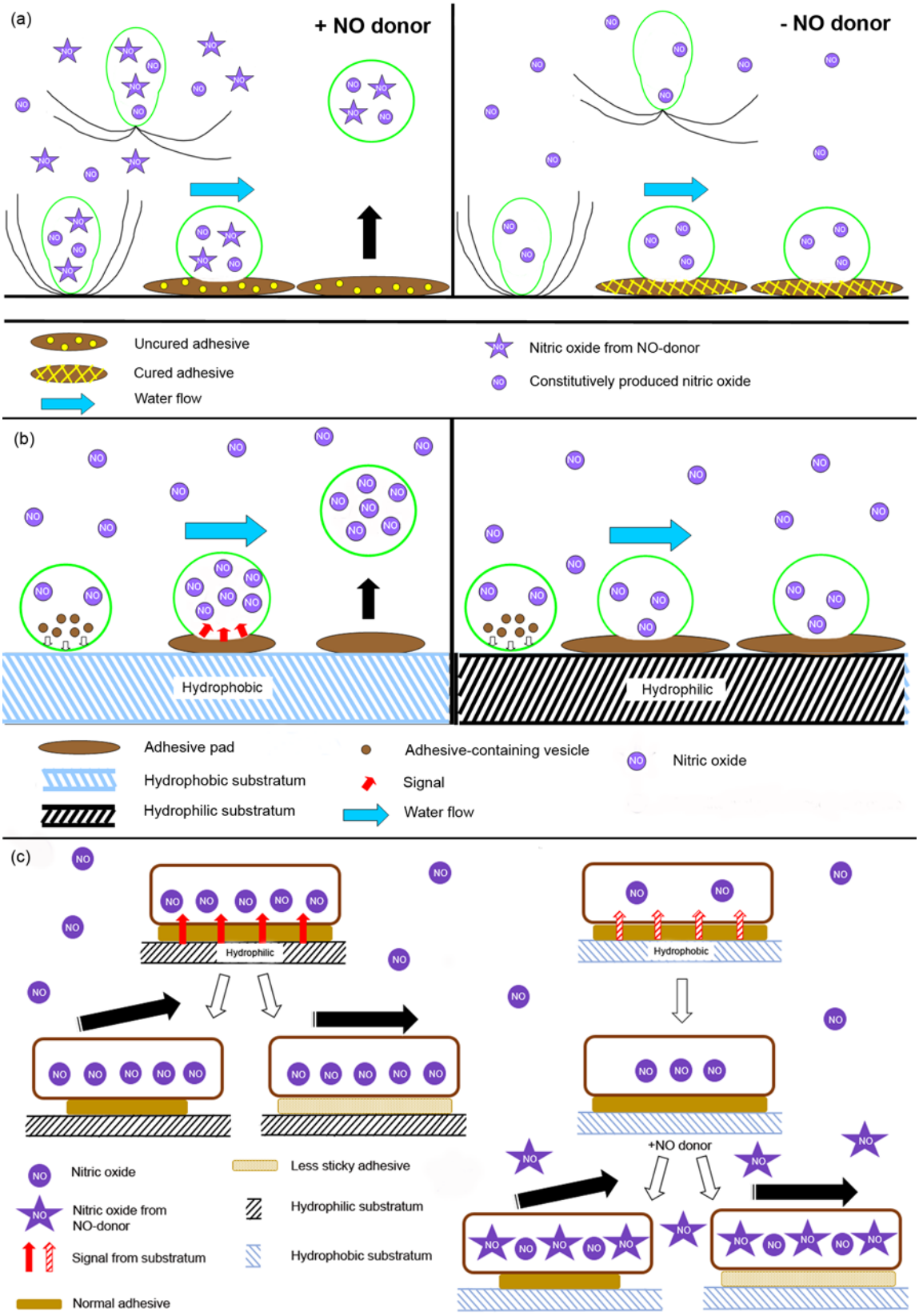
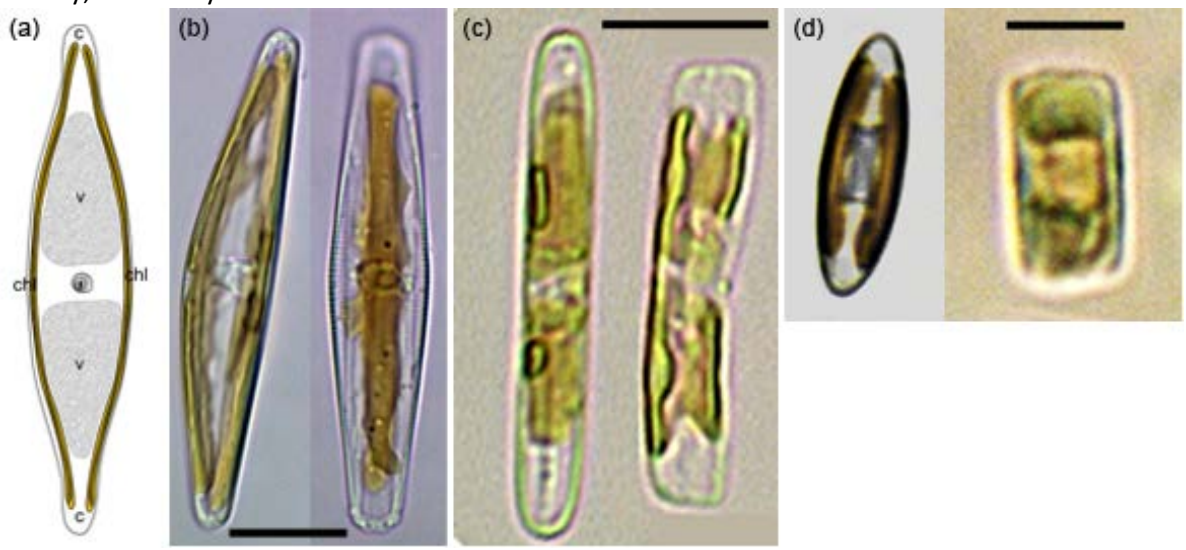

(a)
+ NO donor
- NO donor
Uncured adhesive
Cured adhesive
Water flow
Nitric oxide from NO-donor
Constitutively produced nitric oxide
(b)
Hydrophobic
Hydrophilic
Adhesive pad
Hydrophobic substratum
Hydrophilic substratum
Adhesive-containing vesicle
Signal
Water flow
Nitric oxide
(c)
Hydrophilic
Hydrophobic
+NO donor
Nitric oxide
Nitric oxide from NO-donor
Signal from substratum
Normal adhesive
Less sticky adhesive
Hydrophilic substratum
Hydrophobic substratum

Figure 5 (a) Diagrammatic view of a raphid diatom (reconstructed from an electron micrograph of *Navicula cuspidata* in Edgar & Pickett-Heaps (1982)), illustrating key features. chl = chloroplast, c = cytosol, v = vacuole, n = nucleus. **(b-d)** Light microscope images of the raphid diatoms **(b)** *Seminavis robusta* (bar = 20 µm), **(c)** *Craspedostauros australis* (bar = 20 µm) and **(d)** *Navicula incerta* (bar = 5 µm). The diatoms are shown in both valve view (left) and girdle view (right). Image of *Navicula* courtesy of J.A. Finlay, University of Newcastle.



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Supplementary Information

List of abbreviations used:

AF	antifouling
AHLs	*N*-acylhomoserine lactones
APX	ascorbate peroxidase
AWG	acid washed glass
$[Ca^{2+}]_{cyt}$	cytosolic calcium
CAT	catalase
cGMP	cyclic guanosine monophosphate
DAF-FM	4-amino-5-methylamino-2',7'-dichlorofluorescein
DCFH-DA	dichlorodihydrofluorescein diacetate
DD	2E,4E/2-decadienal
DDT	dichlorodiphenyltrichlorethane
EDTA	ethylenediaminetetraacetic acid
EPS	extracellular polymeric substances
ESTs	expressed sequence tags
FOTS	tridecafluoroctyl-triethoxysilane
GR	glutathione reductase
IP_3	inositol triphosphate
NAADP	nicotinic acid adenine dinucleotide phosphate
NADPH	nicotinamide adenine dinucleotide phosphate
NO	nitric oxide
NOS	nitric oxide synthase
PC	phytochelatins
PDMSe	polydimethylsiloxane elastomer
PEG	polyethylene glycol
QS	quorum sensing
ROS	reactive oxygen species
SAM	self-assembled monolayer
SNAP	*S*-nitroso-*N*-acetylpenicillamine
SNARE	soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor
SOD	superoxide dismutase
TBT	tributyltin